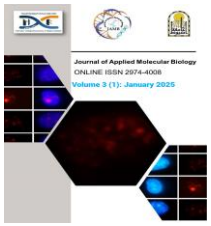
Journal of Applied Molecular Biology (JAMB)
Molecular biology Research & Studies institute , Assuit University, Egypt
ISSN 2974 – 4008
Volume 3 (1): January 2025

https://jamb.journals.ekb.eg



Production of L-asparaginase and L-glutaminase Anticancer Enzymes Using Filamentous Fungi

Radwa M. R. Abdulazem[1], Ahmed M. Moharram[2,3], Abdel-Naser A. Zohri[2], Osama A. M. Al-Bedak[3,4,*]

[1] Molecular Biology Researches Studies Institute Biotechnology Department Assiut University, Assiut 71511, Egypt
[2]Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut, 71511, Egypt
[3]Assiut University Mycological Centre, Assiut University, Assiut, 71511, Egypt
[4] ERU Science & Innovation Center of Excellence, Egyptian Russian University, Badr city, 11829, Cairo, Egypt
*Corresponding Authors: osamaalbedak@science.au.edu.eg

ARTICLE INFO
Article History:
Received: 29-10-2024
Accepted: 21-01-2025
Online: 27-01-2025

Keywords:
Aspergillus; L-asparaginase; L-glutaminase; *Penicillium*; Optimization.

ABSTRACT

Enzymes as therapeutics have advantages over non-enzymatic drugs. Production of L-asparaginase and L-glutaminase using microbial sources is vital biotechnology because of their antileukemic properties. The cost of manufacture and the immunogenicity linked to enzyme medicines are frequently major setbacks to their development. Searching for new microbial sources and maximizing enzyme production are the targeted goals for safe enzyme availability and cost reduction. Therefore, this study focused on screening the ability of 189 fungal isolates related to 9 genera and 27 species to generate L-asparaginase and L-glutaminase. On agar medium, L-asparaginase and L-glutaminase activities were high in 30 and 26 of the positive isolates comprising 17.85 and 16.25 %, respectively. According to the results of the submerged fermentation experiment, the most productive strains for the production of L-asparaginase and L-glutaminase were *Penicillium brevicompactum* AUMC 13014 and *Aspergillus nidulans* AUMC 11446, producing 1086.1 and 438.9 U/mL, respectively. L-asparaginase activity reached its peak (2589.6±250 U/mL) after 5 days of incubation at pH 7.0 and 35 ºC using yeast extract as nitrogen supply. L-glutaminase activity peaked (1364.35±130 U/mL) after 6 days of incubation at pH 6.0 and 20 ºC using ammonium chloride as the nitrogen source. The current study's findings demonstrated the enormous potential of *P. brevicompactum* AUMC 13014 and *A. nidulans* AUMC 11446 as producers of extracellular L-asparaginase and L-glutaminase using submerged fermentation.

1. INTRODUCTION

Due to their remarkable target selectivity and ability to convert multiple substrates, enzymes as therapeutics have advantages over non-enzymatic drugs. Enzyme therapy has increased cancer patients' chances of survival. Metastasis therapy aims to prevent malignant cells from multiplying without normal cells. Amino acid deprivation methodology is a technique used in anti-cancer therapy, in which tumor cells that are auxotrophic to specific amino acids are starved of these amino acids by depleting them. This frequently slows the growth of tumors.

Certain microorganisms, including bacteria, actinomycetes, algae, yeast, and fungi, exhibit the capacity to create stable anticancer chemicals [1]. Fungi are extensively varied microorganisms. Diverse fungi, including terrestrial fungi, endophytes, entomopathogenic fungi, nematode-trapping fungi, coprophilous fungi, and marine and freshwater fungi, exemplify ecological diversity [2]. Fungi are the most significant organisms for biotechnological applications [3]. The production of anticancer enzymes from fungus is expedited, economically viable, scalable, and conducive to genetic modifications [4]. Fungi serve as a compelling source for industrial anticancer enzymes [5]. Fungal anticancer enzymes exhibit excellent production efficiency, simplified purification and separation processes, particularly in filamentous fungus, and effective catalysis with requisite stability under adverse conditions [5]. Fungal cells exhibited enzymatic activity both intracellularly and extracellularly. Fungi are regarded as natural decomposers, thereby enabling them to generate numerous extracellular enzymes essential for the bioconversion of diverse substrates and complexes [6].

The amidase group's L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) catalyzes the hydrolysis of L-asparagine, resulting in the release of L-aspartic acid and ammonia [7,8]. It has demonstrated therapeutic efficacy when used in conjunction with other medications to treat a range of conditions, including melanosarcoma, reticulosarcoma, lymphocytic leukemia, Hodgkin's lymphomas, chronic lymphosarcoma, acute myelocytic leukemia, acute myelocytic leukemia, and acute lymphoblastic leukemia [9-14]. Additionally, it serves as a biosensor to measure asparagine levels during chemotherapy and is utilized in the food business to neutralize the carcinogenic acrylamide [15,16].

Many commercial preparations of L-asparaginases are now used these include Elspar®, Medac, Paronal®, and Kidrolase® from *Escherichia coli*, its pegylated form and *Dickeya dadandii*, and Erwinase® from *Erwinia chrysanthemi* [17-20]. Although formulations of L-asparaginase have greatly improved, adverse effects persist. Even though L-asparaginases from *E. coli* and *E. chrysanthemi* have been used extensively in medicine, more research is needed to find suitable alternatives because of growing issues like hypersensitivity, antigenicity, short half-lives, and transient blood clearance [21-23].

The hydrolysis of L-glutamine to L-glutamic acid and ammonia is catalyzed by L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) [24]. Due to its potential use as an anti-leukemic agent [25-28], in addition to its application in the c-glutamyl transfer processes that yield specialized compounds like theanine, and as a potent antiretroviral drug [29], it has drawn a lot of attention. By selectively denying glutamine-dependent tumor cells of their glutamine, amidases cause neoplasms to lose vital nutrients and

ultimately die [30,31]. The use of L-glutaminase in conjunction with or instead of L-asparaginase may therefore be significant in the context of enzyme therapy for cancer, particularly acute lymphocytic leukemia [29].

This prompted the assignment of this study to screen different collected fungal strains for their potential to produce L-asparaginase and L-glutaminase. Optimization of some fermentation parameters for L-asparaginase and L-glutaminase production by the potent strains has also been targeted.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents:

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical (Sigma-Aldrich, St. Louis, MO) and LOBA CHEMIE PVT.LTD, India.

2.2 Fungal isolates:

A total of 189 fungal isolates representing 27 species of 9 genera were provided from the culture collection of Assiut University Mycological Centre (AUMC), Assiut University (Table S1). These isolates belonging to *Aspergillus* (69 isolates from 10 species), *Penicillium* (68 isolates from 6 species), *Cladosporium* (17 isolates from 2 species), *Talaromyces* (11 isolates from 4 species), *Beauveria bassiana* (8 isolates), *Scopuloriopsis brevicaulis* (8 isolates), *Emericella sydowii* (3 isolates), *Neosartorya clavata* (4 isolates), *Trichoderma koningii* (1 isolate).

Media used in this study:

a. **Malt extract agar (MEA)** [32]**.** It is composed of (g/L): Malt extract powder, 20; Glucose, 20; Peptone, 1.0; Agar, 20.
b. **Czapek's Yeast extract Autolysate agar (CYA)** [32]**.** It composed of (g/L)**:** Sucrose, 30; $NaNO_3$, 2; yeast extract, 5; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$ $7H_2O$, 0.01and Agar, 20.
c. **Sucrose-free Czapek's broth** [32]**:** supplemented with 10 g/L of L-asparagine or L-glutamine or L-methionine. It contained (g/L): Sodium nitrate, 2.0; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005; phenol red (dissolved in ethanol) as an indicator, 0.1; agar 20.0. The final pH was adjusted to 6.2.

All media were sterilized in an autoclave at 121°C for 15 minutes, then cooled to 45°C and poured into 90 mm Petri dishes to solidify. The first two media were used for describing the potent fungal strains, while the third was used as a fermentation medium for screening and optimization of enzymes under investigation.

2.3 Preliminary screening of L-asparaginase and L-glutaminase activity:

A 10 mL of the fermentation medium was poured into the test tubes and the tubes were then sterilized at 121 ºC for 20 min. Afterward, the tubes were inoculated individually with 50 µL of spore suspension (1.8×10^8 spores/mL) obtained from 7-day-old cultures of the tested fungi. The generation of pink color beneath the fungal growth was considered to suggest the development of L-asparaginase or L-glutaminase, due to the release of ammonia attributable to asparagine or glutamine degradation [33].

2.4 Determination of L-asparaginase and L-glutaminase activity in submerged fermentation:

The high L-asparaginase and L-glutaminase producers selected from the previous screening were cultured in 250 mL Erlenmeyer conical flasks each containing 50 mL sucrose-free Czapek's broth. The initial pH was adjusted to 6.2. The inoculated flasks were incubated for 7 days at 30 °C in shacking condition at 150 rpm. After the incubation period, the cell-free supernatant was obtained by centrifugation (10,000 rpm at 4 °C for 10 min) and used as L-asparaginase or L-glutaminase sources [34].

2.5 Assay of L-asparaginase and L-glutaminase activities:

The enzyme assay was done by the Nesslerization method [35]. The reaction mixture containing 0.1 mL of 1.0 % L-asparagine (for asparaginase) or 1.0 % L-glutamine (for glutaminase) + 0.1 mL of an enzyme filtrate. The mixture was incubated at 37 °C for 30 min. Afterward, the reaction was stopped by introducing 0.4 mL of 10.0 % Trichloroacetic acid (TCA). A 0.1 mL of the above mixture was mixed with 0.2 mL of Nessler's reagent and 3.7 mL of distilled water. Absorbance at 480 nm was measured and the amount of released ammonia was determined using ammonium sulfate as standard. One unit of L-asparaginase or L-glutaminase is defined as the amount of enzyme that liberates 1.0 µmol of ammonia at the standard assay conditions according to the following Equation:

$$\textbf{Enzyme activity} = \frac{\text{Absorbance} \times 1000 \times \text{Enzyme DF}}{\mu\text{mol ammonia} \times \text{M.Wt.} \times \text{Time}} \quad \textbf{U/mL}$$

Where: **DF** = dilution factor of the enzyme; **M. Wt.** = molecular weight of asparagine or glutamine. The concentration of proteins was determined according to the method mentioned by Lowry's method [36]. Bovine serum albumin standard curve was used to estimate the protein concentrations.

2.6 Optimizing the fermentation parameters:

Using two factors at the time (TFAT), maximization of the L-asparaginase or L-glutaminase production was studied at pH (3-10), nitrogen sources (ammonium chloride, ammonium sulfate, sodium nitrate, peptone, yeast extract, and beef extract) each at 0.2 %, incubation temperature (20, 25, 30, and 35 °C), and fermentation time (1-7 days). An assay of L-asparaginase and L-glutaminase activities was carried out as previously described.

2.7 Morphological identification of the potent fungi:

On malt extract agar (MEA) and Czapek's yeast extract Autolysate agar (CYA) [32], *Aspergillus nidulans* AUMC 11446 and *Penicillium brevicompactum* AUMC 13014 were inoculated in three-point pattern. Following seven days of incubation at 25 °C, microscopic features were examined with lacto-phenol cotton blue, and the strain employed in this investigation was identified based on both their macroscopic and microscopic characteristics [37,38].

2.8 Statistical analysis:

The data were subjected to two-way ANOVA using the SPSS 19.0 software program. Means and standard deviations were calculated for 5 replicate values. Means were

compared by Duncan´s multiple range test and statistical significance was determined at a 5% level [39].

3. RESULTS

3.1 Preliminary screening of enzyme activity

This test included 189 isolates from nine genera, which corresponded to 27 different fungal species. The tested fungi were categorized as high, moderate, and low asparaginase and glutaminase, producers according to measurements of the pink zone depth beneath the fungal growth (Figures 1&2). Based on the preliminary screening results, 168 and 160 isolates (representing 88.88 and 84.65 % of all isolates, respectively) have been determined to be positive producers of both L-asparaginase and L-glutaminase out of the 189 isolates that were examined for this capacity (Figures 1& 2). Of the positive fungi, 30 and 26 demonstrated high production ability for L-asparaginase and L-glutaminase, respectively. They accounted for 17.85 and 16.25 %, respectively, of isolates that showed positive results (Table 1).

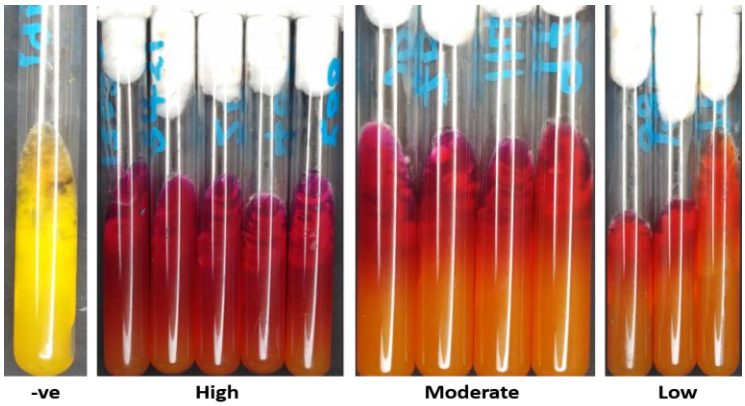


Figure 1. Preliminary screening of L-asparaginase activity of some fungal species on sucrose-free Cz agar medium at 30 ºC (High = ++++; Moderate = ++ and +++; and Low = +).

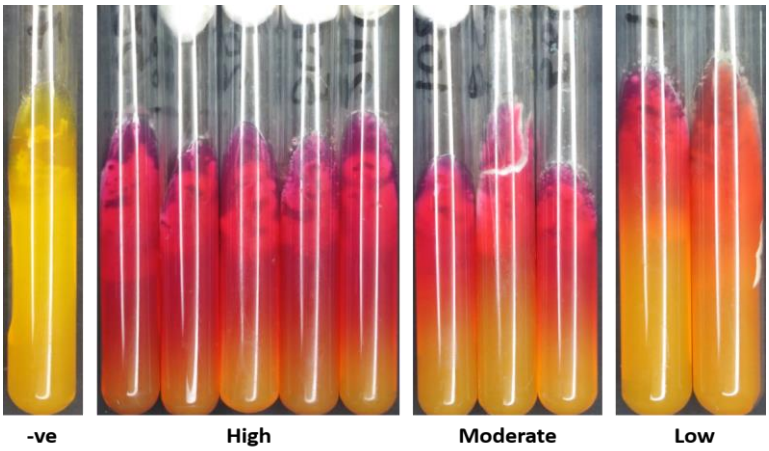


Figure 2. Preliminary screening of L-glutaminase activity of some fungal species on sucrose-free Cz agar medium at 30 ºC (High = ++++; Moderate = ++ and +++; and Low = +).

Table 1. Screening the L-asparaginase and L-glutaminase, activities of 189 filamentous fungal isolates on Sucrose-free Czapek's medium at 30 °C.

Fungal species	No. of isolates	L-asparaginase				L-glutaminase			
		Positive isolates	H	M	L	Positive isolates	H	M	L
Aspergillus	69	51	0	39	12	50	4	37	9
A. brasiliensis	1	0	0	0	0	1	0	1	0
A. carneus	4	4	0	3	1	4	0	3	1
A. flavipes	1	1	0	1	0	1	1	0	0
A. fumigatus	8	8	0	8	0	5	0	3	2
A. melleus	1	1	0	1	0	1	0	1	0
A. nidulans	20	18	0	14	4	20	3	17	0
A. niger	18	3	0	0	3	2	0	0	2
A. ochraceus	3	3	0	2	1	3	0	3	0
A. tamarii	4	4	0	3	1	4	0	0	4
A. terreus	9	9	0	7	2	9	0	9	0
Emericella sydowii	3	3	0	3	0	3	0	3	0
Neosartorya clavata	4	4	0	3	1	2	0	1	1
Penicillium	68	68	26	39	3	67	20	41	6
p. aurantiogriseum	14	14	4	9	1	14	2	9	3
P. brevicompactum	7	7	4	3	0	7	4	3	0
P. chrysogenum	25	25	7	16	2	24	5	17	2
P. corylophilum	4	4	1	3	0	4	4	0	0

Fungal species	No. of isolates	L-asparaginase				L-glutaminase			
		Positive isolates	H	M	L	Positive isolates	H	M	L
P. griseofulvum	5	5	2	3	0	5	2	2	1
P. solitum	13	13	8	5	0	13	3	10	0
Beauveria bassiana	8	8	0	8	0	7	0	5	2
Cladosporium	17	17	3	13	1	17	2	15	0
C. cladosporioides	6	6	1	5	0	6	0	6	0
C. sphaerospermum	11	11	2	8	1	11	2	9	0
Scopuloriopsis brevicaulis	8	8	1	6	1	6	0	5	1
Talaromyces	11	8	0	8	0	7	0	1	6
T. duclauxii	3	3	0	3	0	3	0	0	3
T. funiculosus	1	1	0	1	0	0	0	0	0
T. pinophilus	1	1	0	1	0	0	0	0	0
T. purpureogenus	6	3	0	3	0	4	0	1	3
Trichoderma koningii	1	1	0	1	0	1	0	1	0
Total	189	168	30	120	18	160	26	109	25
No. of genera (9)	9	9	3	9	5	9	3	9	6
No. of species (27)	27	27	9	25	11	25	9	20	13

*Positive enzyme producers, H = high producers (++++); M = moderate producers (++, +++); Low producers (+).

3.2 Secondary screening of L-asparaginase and L-glutaminase activities

To quantify the activity of both enzymes under submerged conditions, assays were conducted on a total of 30 fungal strains that showed high activity for L-asparaginase and 26 of high L-glutaminase producers. L-asparaginase activity ranged from 135.4 U/mL by *P. solitum* AUMC 13593 to 1086.1 U/mL by *P. brevicompactum* AUMC 13014. While the specific activity ranged from 260.1 U/mg protein of *P. solitum* AUMC 13593 to 3419.8 U/mg protein of *P. aurantiogriseum* AUMC 11529. On the other hand, L-glutaminase activity ranged from 131.1 U/mL by *P. brevicompactum* AUMC 498 to 438.9 U/mL by *A. nidulans* AUMC 11446. While the specific activity ranged from 277.9 U/mg protein of *P. chrysogenum* AUMC 507 to 885.6 U/mg protein of *P. solitum* AUMC 13593 (Table 2). As a result, *P. brevicompactum* AUMC 13014 and *A. nidulans* AUMC 11446 were selected for optimizing their fermentation conditions to maximize the L-asparaginase and L-glutaminase production, respectively.

Table 2. Screening of L-asparaginase and L-glutaminase activity in submerged fermentation conditions (SmF) of the highly L-asparaginase and L-glutaminase fungal producers at 30 ºC.

Fungal species	AUMC No.	Asparaginase		Glutaminase	
		Activity U/mL	Specific Activity U/mg	Activity U/mL	Specific Activity U/mg
Aspergillus					
A. flavipes	179	ND	ND	387.7	643.0
A. nidulans	11500	ND	ND	206.8	285.4
A. nidulans	8540	ND	ND	235.6	393.7
A. nidulans	11446	ND	ND	438.9	660.8
Penicillium					
P. aurantiogriseum	11529	954.9	3419.8	206.1	332.2
P. aurantiogriseum	13013	ND	ND	183.7	378.1
P. aurantiogriseum	7044	758.2	1004.6	ND	ND
P. aurantiogriseum	7447	908.8	1581.4	ND	ND
P. aurantiogriseum	330	621.3	880.4	ND	ND
P. brevicompactum	13014	1086.1	1581.4	ND	ND
P. brevicompactum	10583	965.1	1229.5	ND	ND
P. brevicompactum	528	952.1	1152.0	ND	ND
P. brevicompactum	7525	803.6	1086.5	271.7	434.8
P. brevicompactum	498	ND	ND	131.1	286.8
P. brevicompactum	7053	ND	ND	242.8	356.7
P. brevicompactum	5028	ND	ND	311.3	450.3
P. chrysogenum	11559	900.9	1372.0	ND	ND
P. chrysogenum	11556	765.4	1152.4	ND	ND
P. chrysogenum	588	908.8	1757.9	225.5	519.8
P. chrysogenum	2415	711.4	1214.6	304.1	596.1
P. chrysogenum	4033	707.1	1815.5	ND	ND
P. chrysogenum	648	521.8	886.4	ND	ND
P. chrysogenum	5917	652.3	1087.1	ND	ND

Fungal species	AUMC No.	Asparaginase		Glutaminase	
		Activity U/mL	Specific Activity U/mg	Activity U/mL	Specific Activity U/mg
P. chrysogenum	13364	ND	ND	268.2	651.1
P. chrysogenum	507	ND	ND	207	277.9
P. chrysogenum	2024	ND	ND	162.8	315
P. corylophilum	7448	532.6	641.5	262.3	588.1
P. corylophilum	1975	ND	ND	219.8	315.2
P. corylophilum	7049	ND	ND	256.5	636.6
P. corylophilum	2516	ND	ND	225.5	313.9
P. solitum	13593	135.4	260.1	340.9	885.6
P. solitum	13594	904.5	1346.6	252.9	582.9
P. solitum	505	1065.9	1518.7	397.1	707.2
P. solitum	14755	562.2	656.2	ND	ND
P. solitum	15162	630.7	660.5	ND	ND
P. solitum	11566	669.6	1722.6	ND	ND
P. solitum	13588	758.2	1391.4	ND	ND
P. crustosum	3411	583.1	683.6	ND	ND
P. griseofulvum	535	601.1	1531.6	254.4	356.3
P. griseofulvum	534	ND	ND	312.0	554.2
P. griseofulvum	5905	632.8	1255.1	ND	ND
Cladosporium					
C. cladosporioides	11433	531.2	683.3	ND	ND
C. sphaerospermum	136	590.3	774.3	ND	ND
C. sphaerospermum	4440	681.1	1100.5	285.4	709.5
C. sphaerospermum	4448	ND	ND	229.9	322.0
Scopuloriopsis					
S. brevicaulis	435	579.5	1328.3	ND	ND

*ND: None detected

3.2 Optimization of L-asparaginase production by *P. brevicompactum* AUMC 13014

The fermentation medium's pH and temperature were optimized for *P. brevicompactum* AUMC 13014 to maximize the L-asparaginase production. This resulted in the production of L-asparaginase that was active over a wide pH range (3–10) and temperature range (20–35) producing the greatest asparaginase activity of 1441.47±140 U/mL at pH 7 and 35 ºC after 5 days of incubation using sodium nitrate as a nitrogen source (Figure 3). L-asparaginase activity reached its peak of 2589.6±250 U/mL after 5 days of incubation when yeast extract was applied as the nitrogen supply (Figure 4).

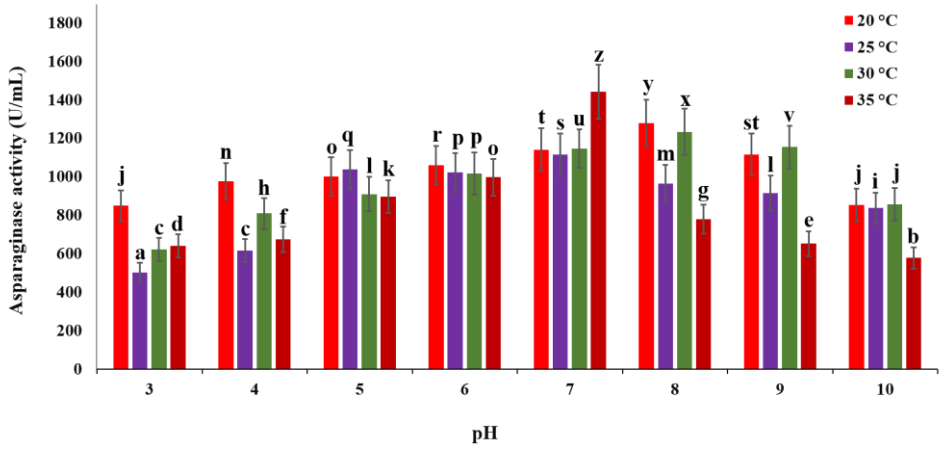


Figure 3. Effect of medium's pH and temperature on L-asparaginase activity produced by *P. brevicompactum* AUMC 13014 after 5 days of incubation using sodium nitrate as a nitrogen source (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

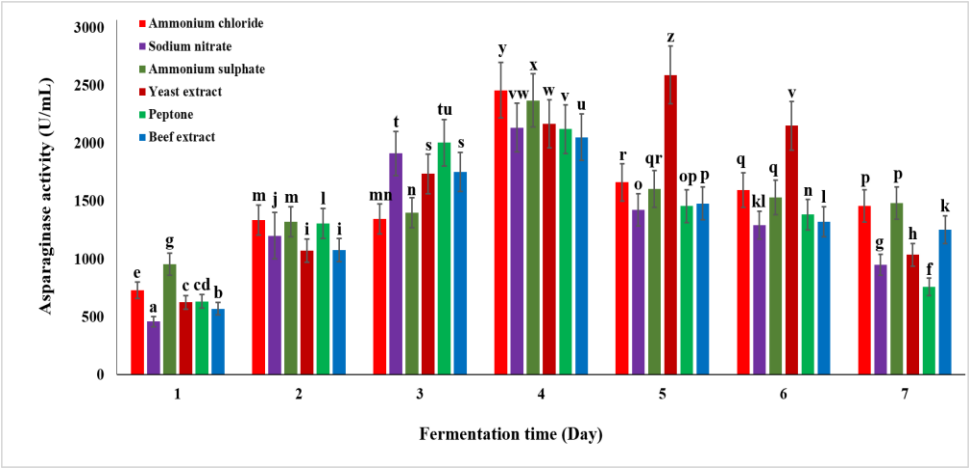


Figure 4. Effect of medium's nitrogen source and fermentation time L-asparaginase activity produced by *P. brevicompactum* AUMC 13014 (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

3.3 Optimization of L-glutaminase production by *A. nidulans* AUMC 11446

To increase the synthesis of L-glutaminase, the pH and temperature of the fermentation medium were altered for *A. nidulans* AUMC 11446. After 5 days of incubation using sodium nitrate as a nitrogen source, this produced L-glutaminase that was active across a broad pH spectrum (3–10) and temperature range (20–35), producing the maximum glutaminase activity of 939.12±85 U/mL at pH 6 and 20 ºC (Figure 5). Using ammonium chloride as the nitrogen source, L-glutaminase activity peaked after 6 days of incubation at 1364.35±130 U/mL (Figure 6).

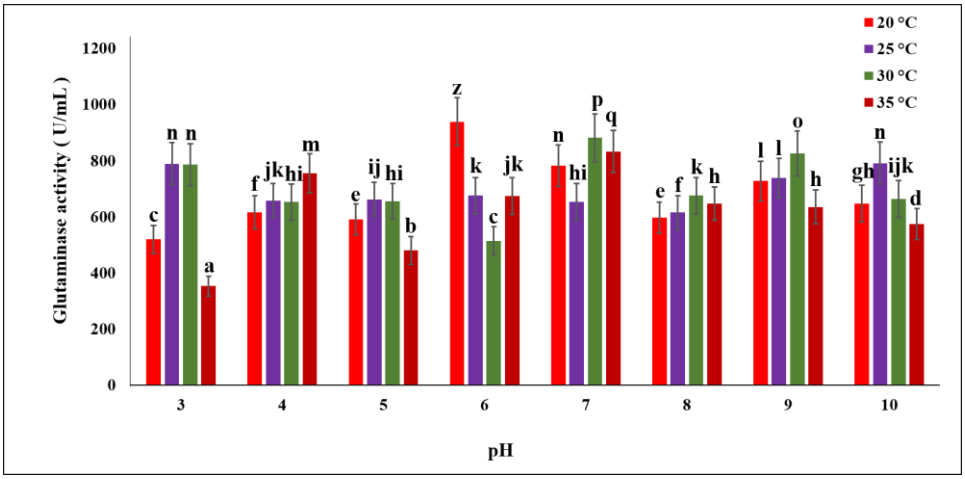


Figure 5. Effect of medium's pH and temperature on glutaminase activity produced by *A. nidulans* AUMC 11446 (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

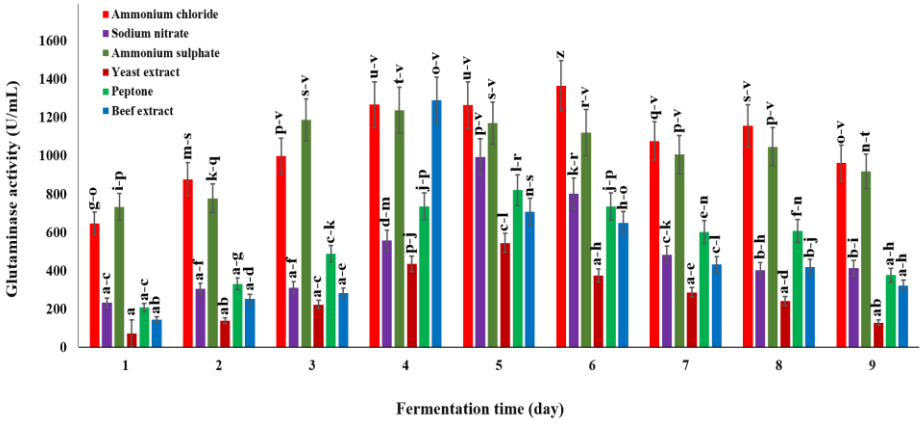


Figure 6. Effect of medium's nitrogen source and fermentation time on glutaminase activity produced by *A. nidulans* AUMC 11446 (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

3.4 Brief description of the potent fungi

3.4.1 Aspergillus nidulans AUMC 11446

Colonies attaining a diameter of 3.5-4.5 cm on malt extract agar after 7 days at 25 ºC. Cleistothecia solitary, globose. up to 150 µm in diameter, surrounded by scattered yellowish hyphae bearing Hülle cells; asci 8-ascospores; ascospores red, lenticular, convex walls smooth, with two equatorial ridges, spore body in face view 4.3-5.1 µm in diameter, in side view 4.6-5.7 × 3.4-4.0 µm, ridges entire. Conidiophores sinuous, smooth, brownish, up to 100 µm long; conidial heads short columnar; vesicles hemispherical, 6-10 µm in diameter; metulae 6.5-7.5 × 2.5-3.3 µm, each bearing 2-3 phialides; phialides 5.8-6.7 × 2.5-3.0 µm; conidia globose, smooth to finely roughened, 2.5-4.2 µm in diameter (Figure 7).

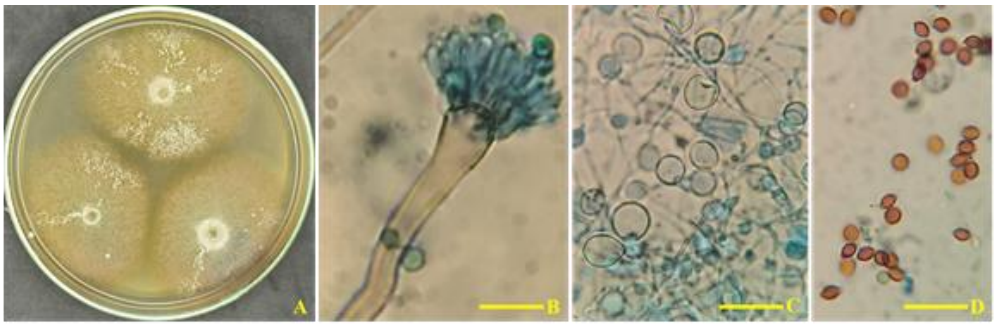


Figure 7. *Aspergillus nidulans* AUMC 11446. (A) Seven-day-old colonies on MEA at 25 ºC. (B) Short, brown, biseriate, columnar conidiophore. (C) Hülle cells. (D) Ascospores (Scale bar = 20 µm).

3.4.2 Penicillium brevicompactum AUMC 13014

Colonies attaining 20–30 mm in diameter after 7 days at 25 ºC, surface texture typically velutinous. Margins white. Conidiogenesis is light to moderate, commonly Dull Green. Exudates are usually present. Conidiophores are usually long and broad, 500-800×4.0-6.0 µm, smooth-walled, characteristically bearing compact, broad terverticillate penicilli. Metulae short and board, 9-12×3.5-5.0 µm. Phialides ampulliform, 6-7×2.5-3.0 µm. Conidia smooth to very finely roughened, commonly ellipsoidal, 2.5-3.5 × 2.0-2.5 µm (Figure 8).

Figure 8. *Penicillium brevicompactum* AUMC 13014. (A) Seven-day-old colonies on CYA at 25 ºC. (B–C). Conidiophores bearing compact, broad terverticillate penicilli (D) Conidia (Scale bar = 20 µm).

4. DISCUSSION

Commercial formulations of *Erwinia chrysanthemi* and *Escherichia coli* asparaginases have been shown to exhibit cross-reactivity with glutamine and urea. Accordingly, new microbiological sources of asparaginase need to constantly be investigated. Finding a different microbial source of L-asparaginase and L-glutaminase production was, thus, the aim of the present study.

The potential of 189 isolates from 27 different fungal species from nine genera to produce L-asparaginase and L-glutaminase on agar medium and in submerged fermentation was examined in this work. It has been found that 88.36 and 84.65% of all isolates, respectively, were positive producers of L-asparaginase and L-glutaminase.

Many fungal species, including *Aspergillus*, *Trichoderma*, *Chaetomium*, *Alternaria*, *Curvularia*, *Pleurotus*, *Fusarium*, *Pestalotiopsis*, *Penicillium*, *Drechslera*, *Cladosporium*, *Phoma*, and *Phomopsis* species, are distributed carriers of the anti-proliferative enzyme L-asparaginase [40-45].

Compared to our results, Kumar [46] screened ten fungal isolates to determine their potential to produce L-asparaginase in SmF. Twenty percent of the fungi they investigated were found to be able to produce L-asparaginase with *Fusarium* sp. SMGR-F1 is the most potent strain, generating 111.07±1.53 U/mL of L-asparaginase after 120 hours. After screening a variety of soil fungi for asparaginase activity, Rani [47] determined that the most potent strain of *Aspergillus* sp. was KUFC 20, producing 70.67±1.14 U/g of asparaginase activity. The capacity of *Lasiodiplodia theobromae* SCUF-TP2016 to generate L-asparaginase was assessed both qualitatively and quantitatively. After Sephadex G 100 was used to partially purify the enzyme, it yielded 21 U/mL of asparaginase [48].

Nutritional and physicochemical factors have a significant influence on most extracellular enzymes produced by the microorganism. Optimizing the different media components in a process that relies on microorganisms producing biologically active compounds can significantly affect production costs and yield a profit or loss. Investigating the appropriate conditions for biotechnology solutions is therefore essential. The current results revealed that *P. brevicompactum* AUMC 13014 was the potent L-asparaginase producer. After optimizing the fermentation parameters, it produced the maximum L-asparaginase of 2589.6±250 U/mL after 5 days of incubation at pH 7.0 and 35 ºC when yeast extract was applied as the nitrogen supply. This study's asparaginase activity was significantly higher than that of *Fusarium equiseti* AHMF4 (40.78 U/mL) which was obtained after 7 days at pH 7.0 and 30 ºC [49]. On the fifth day of incubation, the optimal conditions for maximum production of L-asparaginase under SSF by *Fusarium solani* AUMC 8615 were reached at 30 °C, pH 8.0 [50]. *Fusarium foetens* produced the highest L-asparaginase activity of 12.83 U/mL at pH 8.0 and 27.5 ºC after 7 days [51]. *A. fumigatus* produced the highest L-asparaginase activity (23.83 U/mL) at pH 8 and 40 ºC [37]. *A. terreus* CCT 7693 exhibited the L-asparaginase activity peak (13.81 U/mL) at pH 9.49 and 34.6 ºC [52]. *F. culmorum* ASP87 and *F. brachygibbosum*-ASP56 displayed their maximum L-asparaginase activity at pH 7.5 and 6.0, and temperature of 30 ºC [53].

The current study adjusted the fermentation conditions of *A. nidulans* AUMC 11446 to produce L-glutaminase. After 6 days at pH 6.0 and 20 ºC, the strain was able to produce 1364.35±130 U/mL of L-glutaminase with ammonium chloride serving as the nitrogen source. L-glutaminase production conditions have been screened and optimized using a variety of filamentous fungi. Regarding this concern, the highest amount of L-glutaminase (49.89 U/mL) was produced after four days of incubation at pH 9.0 and 27 °C by *Beauveria* sp. [54]. After incubating for seven days at pH 7.0 and 30 ºC, *Trichoderma koningii* showed the maximal L-glutaminase activity of 23.2 U/mg protein under solid-state conditions [55]. *Penicillium brevicompactum* NRC 829 showed the highest L-glutaminase activity (7.4 U/mL) after 4 days at pH 6.0 and 28 ºC utilizing sodium nitrate as a nitrogen source [31]. The maximal activity of *Hypocrea jecorina* L-glutaminase, 13.75 U/mL, has been determined at pH 8.0 and 50 ºC [56]. The maximum

yield of enzyme production (2777 U/mL) by *Fusarium oxysporum* was achieved at pH 6.0 and 35 °C after 7 days [57].

CONCLUSION

The current study focused on finding novel sources from filamentous fungi that produce L-asparaginase and L-glutaminase while also maximizing the fermentation characteristics of the strongest strains. The strains *P. brevicompactum* AUMC 13014 and *A. nidulans* AUMC 11446 were shown to be highly effective in producing L-asparaginase and L-glutaminase, respectively. Additionally, the ideal circumstances for producing both enzymes were ascertained.

Supplementary material: Table S1. Fungal genera and species given with their AUMC accession numbers alongside with sources of isolation

REFERENCES

1. S. Nobili, D. Lippi, E. Witortc, M. Donninic, L. Bausi, E. Minia, S. Capacciolic, Natural compounds for cancer treatment and prevention, Jornal of Pharmacological Research, 59 (2009) 378.
2. J. Collado, G. Platas, F. Pelaez, Identification of endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus, Jornal of Mycologi, 93 (2001) 886.
3. C. P. Kurtuzman, Fungi – source of food, fuel and biochemicals, Jornal of Mycologia, 75 (1983) 382.
4. R. S. Singh, T. Singh, A. Pandey, Microbial enzymes an overview, Advances in enzyme technology, (2019) 40.
5. N. Kango, U. K. Jana, R. Choukade, Fungal enzymes, Sources and biotechnological applications, Advancing Frontiers in Mycology and Mycotechnology, Basic and Applied Aspects of Fungi, (2019) 538.
6. M. L. Berbee, T. Y. James, C. Strullu-Derrien, Early diverging fungi, Diversity and impact at the dawn of terrestrial life, Annual Review of Microbiology, 71(1) (2017) 60.
7. K. Kumar, N. Verma, The various sources and application of L-asparaginase, Asian Journal of Biochemical and Pharmaceutical Research, 2(3) (2012) 197.
8. H. Saeed, A. Hemida, N. El-Nikhely, M. Abdel-Fattah, M. Shalaby, A. Hussein, H. Nematalla, Highly efficient *Pyrococcus furiosus* recombinant L-asparaginase with no glutaminase activity: Expression, purification, functional characterization, and cytotoxicity on THP-1, A549 and Caco-2 cell lines, International Journal of Biological Macromolecules, 156 (2020) 812.
9. N. Verma, K. Kumar, G. Kaur, S. Anand, L-asparaginase: A promising chemotherapeutic agent, Critical Reviews in Biotechnology 27(1) (2007) 45.

10. N.E. El-Naggar, M.E. Sara, M.E. Nancy, Microbial L-asparaginase as a potential therapeutic agent for the treatment of acute Lymphoblastic Leukemia: the pros and cons, International Journal of Pharmacology 10(4) (2014) 182.
11. U. Ali, M. Naveed, A. Ullah, K. Ali, S.A. Shah, S. Fahad, A.S. Mumtaz, L-asparaginase as a critical component to combat Acute Lymphoblastic Leukaemia (ALL): A novel approach to target ALL, European Journal of Pharmacology, 771 (2016) 199.
12. I. Husain, A. Sharma, S. Kumar, F. Malik, Purification and characterization of L-glutaminase free L-asparaginase from enterobacter *Cloacae*: in-vitro evaluation of cytotoxic potential against human myeloid leukemia HL-60 cells, Journal Placement and Citation Performance, 11(2) (2016), e0148877
13. R. Shi, Y. Liu, Q. Mu, Z. Jiang, S. Yang, Biochemical characterization of a novel L-asparaginase from *Paenibacillus barengoltzii* being suitable for acrylamide reduction in potato chips and mooncakes, International Journal of Biological Macromolecules, 96 (2017) 93.
14. J.L. Krleza, A.K. Bojanac, G. Jakovljevic, Determination of L-asparaginase activity and its therapeutic monitoring in children with hematological malignancies in a single Croatian Center, Diagnostics 14(6) (2024) 623.
15. A. Emadi, J.Y. Law, E.T. Strovel, R.G. Lapidus, L.J. Jeng, M. Lee, M.G. Blitzer, B.A. Carter-Cooper, D. Sewell, I. Merwe, Aspaaginase *Erwinia chrysanthemi* effectively depletes plasma glutamine in adult patients with relapsed refractory acute Myeloid Leukemia, Cancer Chemotherapy and Pharmacology 81 (2018) 217.
16. C. Zuzana, K. Kukurová, Impact of l-asparaginase on acrylamide content in fried potato and bakery products, In: V. Gökmen, B.A. Mogol, Acrylamide in Food (2nd Edition). Academic Press (2024) 473.
17. R. Samson, J.B. Legendre, R. Christen, M.F. Saux, W. Achouak, L. Gardan, Transfer of *pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen, International Journal of Systematic and Evolutionary Microbiology 55(4) (2005) 1415.
18. V.D. Berg, Henk, Asparaginase revisited, Leukemia and Lymphoma 52(2) (2011) 168.
19. Y. Wang, W. Xu, H. Wu, W. Zhang, C. Guang, W. Mu, Microbial production, molecular modification and practical application of L-asparaginase, International Journal of Biological Macromolecules 186 (2021) 975.
20. S. Darnal, V. Patial, V. Kumar, S. Kumar, V. Kumar, Y.S. Padwad, D. Singh, Biochemical characterization of extremozyme L-asparaginase from *Pseudomonas* sp. PCH199 for therapeutics., AMB Express 13(1) (2023) 22.
21. A.H. Nguyen, Y. Su, J.Y. Zhang, A. Antanasijevic, M. Caffrey, A.M Schalk, L. Liu, D. Rondelli, A. Oh, D.L. Mahmud, A novel L-asparaginase with low L-glutaminase coactivity is highly efficacious against both T-and B-cell Acute Lymphoblastic Leukemias *in vivo*, Cancer Research 78(6) (2018) 1549.

22. A. Radadiya, W. Zhu, A. Coricello, S. Alcaro, N.G Richards, Improving the treatment of acute Lymphoblastic Leukemia, Biochemistry 59(35) (2020) 3193.
23. M. Sobat, S. Asad, M. Kabiri, M. Mehrshad, Metagenomic discovery and functional validation of L-asparaginases with anti-leukemic effect from the Caspian sea, Iscience 24(1) (2021).
24. P.V. Iyer,V. Padma, R.S. Singhal, Screening and selection of marine isolate for L-glutaminase production and media optimization using response surface methodology, Applied Biochemistry and Biotechnology 159 (2009) 233.
25. J. Roberts, J.S. Holcenberg, W.C. Dolowy, Antineoplastic activity of highly purified bacterial glutaminases, Nature 227.5263 (1970) 1136-1137.
26. J. Roberts, Purification and properties of a highly potent antitumor glutaminase-asparaginase from *Pseudomonas* 7Z, Journal of Biological Chemistry, 251(7) (1976) 2119.
27. S.R. Kumar, M. Chandrasekaran, Continuous production of L-glutaminase by an immobilized marine *Pseudomonas* sp. BTMS-51 in a packed bed reactor, Process Biochemistry 38(10) (2003) 1431.
28. L. Kaushal, K. Alka, E. Arya, D. Chand, Production, Purification and Characterization of L-glutaminase from *Pseudomonas* sp. GPB-06, International Journal of Technology and Engineering Studies, 9(1) (2023) 1.
29. R. Nandakumar, K. Yoshimune, M. Wakayama, M. Moriguchi, Microbial glutaminase: Biochemistry, molecular approaches and applications in the food industry, Journal of Molecular Catalysis B: Enzymatic 23(2) (2003) 87.
30. S. Pal, Antineoplastic activities of purified bacterial glutaminase on transplanted tumour system, Indian Journal of Cancer Chemotherapy 13 (1992) 73.
31. A.M. Elshafei, M.M. Hassan, M.E.Ali, D.A. Mahmoud, D.H. Elghonemy, Screening and optimization of L-asparaginase and L-glutaminase production by some filamentous fungi, Advances in Food Sciences 34(3) (2012) 150.
32. D. Smith, A.H.S. Onions, The preservation and maintenance of living fungi, Commonwealth Agricultural Bureaux, Slough, UK (1994).
33. M. Wakayama, T. Yamagata, A. Kamemura, N. Bootim, S. Yano, T. Tachiki, K. Yoshimune, M. Moriguchi, Characterization of salt tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation, Journal of Industrial Microbiology and Biotechnology, 32(2005) 390.
34. R. Gulati, R. K. Saxena, R. Gupta, A rapid plate assay for screening l-asparaginase producing micro-organisms, Letters in applied microbiology, 24(1) (1997)26.
35. A. Imada, S. Igarasi, K. Nakahama, M. Isono, L-asparaginase and L-glutaminase activities of microorganisms, Microbiology 76(1) (1973) 85.
36. O. Lowry, N. Rosebrough, A.L. Farr, R. Randall, Protein measurement with the folin phenol reagent, Journal of Biological Chemistry 193(1) (1951) 265.

37. A.H. Moubasher, Soil fungi in Qatar and other Arab countries, 1st Edition, Scientific and Applied Research Center, Qatar University, Doha, Qatar (1993) 566.

38. J.I. Pitt, The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*, 1st Edition, Academic Press INC, London, United kingdom, (1979) 634.

39. L. Stahle, S. Wold, Analysis of variance (ANOVA), Chemometrics and Intelligent Laboratory Systems, 6(4) (1989) 259.

40. M.M.A.A. El-Gendy, A. Nageh, T. Taher, H. Fareed, Production, purification and characterization of L-asparaginase from marine endophytic *Aspergillus* sp. ALAA-2000 under submerged and solid state fermentation, Journal of Microbial and Biochemical Technology 7(3) (2015) 165.

41. M.M.A.A. El-Gendy, S.H.M. Al-Zahrani,A.M.A. El-Bondkly, Construction of potent recombinant strain through intergeneric protoplast fusion in endophytic fungi for anticancerous enzymes production using rice straw, Applied Biochemistry and Biotechnology 183 (2017) 30.

42. M.M.A.A. El-Gendy, S.M. Yahya, A.R. Hamed, M.M. Soltan, A.M.A. El-Bondkly, Phylogenetic analysis and biological evaluation of marine endophytic fungi derived from Red sea sponge *Hyrtios erectus*. Applied Biochemistry and Biotechnology 185 (2018) 755.

43. D. Benchamin, R. Sreejai, L. Athira, F.J. Roshan, S. Sujitha, B.S. Kurup, Production and characterization of L-asparaginase isolated from *Aspergillus fumigatus*, The Pharma Innovation Journal 8(3) (2019)a 220.

44. C. Pallem, Solid-state fermentation of corn husk for the synthesis of L-asparaginase by *Fusarium oxysporum*, Asian Journal of Pharmacy and Pharmacology 5(4) (2019) 678.

45. A.A. Prihanto, I.O. Caisariyo, K.A. Pradarameswari. *Aspergillus* sp. as a potential producer for L-asparaginase from mangrove (*Avicennia germinans*), IOP Conference Series: Earth and Environmental Science (2019) 230

46. R. Kumar, V.K. Sedolkar, A. Triveni, M.S. Kumar, C.T. Shivannavar, S.M. Gaddad, Isolation, screening and characterization of L-asparaginase producing fungi from medicinal plants, International Journal of Pharmacy and Pharmaceutical Sciences 8(1) (2016) 281.

47. S.A. Rani, L. Sundaram, P.B. Vasantha, Isolation and screening of L-asparaginase producing fungi from soil samples, International Journal of Pharmacy and Pharmaceutical Sciences, 4(1) (2012) 279.

48. B.A. Balbool, A. Ahmed, M. Moubasher, E. Helmy, Production of L-asparaginase (L-ASN) from endophytic *Lasiodiplodia theobromae* hosted *Teucrium polium* in Egypt. Microbial Biosystems 3(2) (2018) 46.

49. M.M.A.A. El-Gendy, M.F. Awad, F.S El-Shenawy, A.M.A. El-Bondkly, Production, purification, characterization, antioxidant and antiproliferative activities of

extracellular L-asparaginase produced by *Fusarium equiseti*, Saudi Journal of Biological Sciences 28(4) (2021) 2540.

50. G.S. Isaac, M. A. Abu-Tahon, Production of extracellular anti-leukemic enzyme L-asparaginase from *Fusarium solani* AUMC 8615 grown under solid state fermentation conditions: purification and characterization of the free and immobilized enzyme, Egyptian Journal of Botany 56(3) (2016) 799-816.
51. J. Parashiva, B.R. Nuthan, M. Bharatha, R. Praveen, P. Tejashwini, S. Satish, Response surface methodology based optimized production, purification, and characterization of L-asparaginase from *Fusarium foetens*, World Journal of Microbiology and Biotechnology 39(9) (2023) 252.
52. T.A. Costa-Silva, D.I. Camacho-Córdova, G.S Agamez-Montalvo, L.A. Parizotto, I. Sánchez-Moguel, A. Pessoa-Jr, Optimization of culture conditions and bench-scale production of anticancer enzyme L-asparaginase by submerged fermentation from *Aspergillus terreus* CCT 7693, Preparative Biochemistry and Biotechnology 49(1) (2019) 95.
53. A.K. Meghavarnam, S. Janakiraman, Optimization of physiological growth conditions for maximal production of L-asparaginase by *Fusarium* species, International Journal of Bioassays 4 (2015) 4369.
54. M. Chandrasekaran, S.R. Kumar, T. Keerthi, A. Sabu, L-glutaminase production by marine *Beauveria* sp., under solid state fermentation. Process Biochemistry 35 (1999).
55. A.S. El-Sayed, L-glutaminase production by *Trichoderma koningii* under solid-state fermentation, Indian Journal of Microbiology 49 (2009) 243.
56. D. Bülbül, E. Karakuş, Production and optimization of L-glutaminase enzyme from *Hypocrea jecorina* pure culture, Preparative Biochemistry and Biotechnology 43(4) (2013) 385.
57. S.R. Hamed, R.S. Al-wasify, Production and optimization of L-glutaminase from a terrestrial fungal *Fusarium oxysporum*, International Journal of PharmTech Research 9(4)(2016) 233.